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Ethanol Extract of Young Papaya Seeds (*Carica Papaya*, L) Lower The Level of Testosterone, Spermatozoa Count and Expression of Androgen Receptors in Sertoli Cells Of Adult Mice (Mus Musculus)

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Ethanol extract of young papaya seeds (*Carica papaya L.*) contains steroids that thought to has the antifertility property. This study aims to prove this hypothesis by measuring the low levels of testosterone hormone, spermatozoa count, and androgen receptor expression in sertoli cells. We use the randomized post-test only control group design on thirty-six male Balb/c strain adult male mice aged 12-14 weeks, weight 20-25 grams. They were randomly divided into a control and treatment group. The treatment group was given the same food as the control group plus ethanol extract of young papaya seeds 20 mg/20 gram body weight as much as 0.5 ml, orally every day for thirty-six days. Data were analyzed using the independent-sample T-test and Mann Whitney test. The levels in the treatment group were significantly ($p<0.05$) lower than those in the control. Testosterone levels 31,64±1,91 vs 48,67±1,81 nmol/L, spermatozoa count 42,72±3,33 vs 75,89±4,71 cell/field of view, and androgen receptor expression 28,11±3.06% vs 55.07±2.49%. The ethanol extract of young papaya seeds 20 mg/20 g body weight has the antifertility property.

Keywords: Ethanol extract of young papaya seeds, Testosterone hormone, Spermatozoa, androgen receptors, sertoli cells.

The Indonesian government deals with uncontrolled population growth via its Family Planning (KB) program. The implementation of family planning aims to inhibit or postpone pregnancy for various reasons, including planning for pregnancy, limiting the number of children, and avoiding the medical risks of pregnancy[1].

Active participation of men in family planning in developing countries other than Indonesia varies, for example, Bangladesh 8%, Nepal 24%, and Malaysia 16.8% according to the 2007-2008 SDKI (Indonesian Demographic Health Survey)[2].

Family planning programs require the participation of various parties, both men and women. However, the participation of men in family planning is lacking because in the family planning product and service domain,

the contraceptives are aimed solely for women as the primary target compared to contraception for men. It means that, men have few options for contraception compared to women. Existing male contraceptive methods consist of interrupted intercourse (*coitus interruptus),* condoms, and vasectomy. The interrupted intercourse (*coitus interuptus)* is the oldest natural contraceptive method performed by ejaculating sperm outside the vagina during a fertile period[3].

Research on plants that have the antifertility properties developed by gathering information from the knowledge of traditional medicine[4]. There are various types of plants that can be used as traditional medicine. The papaya plant (*Carica papaya L.*) contained broad spectrum of phytochemicals, including polysaccharides, vitamins, minerals, enzymes, proteins, alkaloids, glycosides, fats and oils, lectins, saponins, flavonoids, steroids, etc[5].

Satriyasa & Pangkahila reported that methanol fraction and n-hexane fraction of young papaya seeds could inhibits spermatogonia development in male mice. Based on this background, we prepared an experiment to investigate whether an ethanol-based extract of young papaya seeds (*Carica papaya L.*) at a level of 20 mg/20g of body weight lower testosterone levels, sperm count and the expression androgen receptors in sertoli cells in adult mice (*Mus musculus*) compared to a control group[6].

MATERIAL AND METHOD

This is an experimental study using a randomized post-test-only control group design, conducted from July 2018 to February 2019. We use adult male mices (*Mus musculus*) as experimental animals.

The study has received ethical clearance from the ethics committee of the faculty of medicine, Udayana University No: 1955/UN 14.2.2.VII.14/LP/2018, dated on September 3rd, 2018, and has received research implementation approval from the Integrated Biomedical Lab Unit, No: 403/UN 14.2.2VII.6/LT/2018.

Young papaya seeds *(Carica papaya L.)* were obtained from farms in the Cau hamlet area, Tua Village, Marga District, Tabanan Regency, Bali. The extraction process was carried out by the maceration method with 96% ethanol solvent. Three thousand gram dried young papaya seeds *(Caricapapaya L.)* produced from 1000 grams of dried organic matter. This organic matter was immersed in ethanol or five days and was stirred every 6 hours. Then, the solution was filtered, and the pulp was macerated again with a 25% mixture (2.5 liters) of solvent and macerated again with the same process. Thin Layer Chromatography (TLC) tests were conducted at UGM Integrated Testing and Research Laboratory. The qualitative test of the ethanol extract of young papaya seed (*Carica papaya L.*) shows a positive result for the presence of steroids.

The ethanol extraction of young papaya seeds was managed and performed at the Pharmacy Laboratory, Faculty of Medicine, Udayana University. The maintenance and treatment of the mices were carried out at the Laboratory Animal Unit, Pharmacology Division, Faculty of Medicine, Udayana University. Immunohistochemical (IHC) examination of androgen receptors on the sertoli cells was carried out in the Integrated Biomedical Laboratory of Faculty of Medicine, Udayana University and the Histology Division of the Faculty of Medicine, Udayana University. The testosterone and spermatozoa examination was carried out at the Reproductive or Anatomy Laboratory, Faculty of Veterinary Medicine, Udayana University.

We study on adult male mices (*Mus musculus*) aged 12-14 weeks, Balb/c strain, weighing 20-25 grams, from one offspring, healthy and not physically disabled. They were obtained and maintained at the Pharmacology Laboratory, Faculty of Medicine, Udayana University, following the principles of 3R, among others[7]. The total samples were 36 male mices, divided randomly and equally into the treatment and control group. The animals were kept in a 40x30x15 cm cage with a 12/12 hour light-dark cycle (dark from 8 pm to 8 am and light from 8 am to 8 pm). The animals were given free access to food and sufficient water (*ad libitum*). The treatment group was given ethanol extract of young papaya seeds with levels of steroids, flavonoids of 20 mg/20 g body weight, 0.5 ml, every day for 36 days, before treatment the mice were adapted for one week; on day 37, blood samples were taken from the mices for hormone

testing, sperm was collected for spermatozoa count, and testicular tissue was taken for examination of androgen receptor expression.

Data normality was tested using the Shapiro-Wilk test because of the small sample size. P-value>0.05 indicates the data is normally distributed; in contrast, p-value<0.05 indicates the data is abnormally distributed (skewed). Comparative tests were used to determine differences in the levels of testosterone, spermatozoa count, and number of androgen receptors in sertoli cells in both groups. It was achieved using an unpaired t-test if the distribution of data was normal or a Mann-Whitney test if the data is abnormally distributed.

RESULTS

Variable levels testosterone, spermatozoa, and androgen receptor expression in sertoli cells in the treatment and control groups were all plotted on a numeric scale so that data normality tests could be performed as a condition of bivariate analysis (Table 1). Data is normally distributed if the p-value>0.05.

Table 2 shows the differences between testosterone levels in the treatment and control groups. The mean levels of testosterone in the treatment group were lower than the control group following the daily administration of the ethanol extract of young papaya seed for 36 days. The mean difference between the two groups was 17.02 %, with a p-value<0.001.

Table 3 and figure 1 illustrate the differences between the number of spermatozoa in the control and treatment groups. The number of spermatozoa in the treatment group is lower than that of the control group, following the daily administration of ethanol extract of young papaya seed for 36 days. The mean difference between the two groups was 33,17, with a p-value<0.001.

The androgen receptor expression in the sertoli cells was measured by IHC on testicular tissue using the Rabbit Anti-phospho-Androgen Receptor Polyclonal Antibody (Ser578) kit. Alight microscope connected to *Optilab,* with a 400x magnification (40x objective and 10x ocular) was used.

The results of the IHC examination of the androgen receptor expression in the sertoli cells

Table 1. Normality test of testosterone, spermatozoa and androgen receptor expression in sertoli cells in both groups

Variables	Group	Shapiro-Wilk		
		Statistics	df	Sig.
Testosterone	Treatment	0.93	18	0.23*
	Control	1,00	18	1,00*
Spermatozoa	Treatment	0.96	18	0.61*
	Control	.97	18	0.87*
Androgens Receptor	Treatment	0.92	18	0.14*
Expression in Sertoli cells	Control	0.94	18	0.23*

*p-value<0.05= significant

Table 2. Differences in the testosterone levels between the two groups

Variable	Group	Average SD	Median Min-Max	Average difference	CI 95%	p-value
Testosterone	Control	48.67±1.81	48.66 45.01±52.35	17.02	15.76-18.29	<0.001*
	Treatment	31.64±1.91	31.36 28.69±35.28			

*p-value <0.05= significant

of mice in the control group and the treatment group over the 36-daytest period are presented in table 4 and figure 2.

Figure 2 shows that in the treatment group, the appearance of androgen expression (brown color cell nucleus) was less than in the control group. The green arrow indicates a sertoli cell whose cell nucleus has expressed androgens, whereas the red arrow indicates a sertoli cell whose cell nucleus has not expressed androgens.

DISCUSSION

Ethanol extract of young papaya seeds (*Carica papaya L.*) lower the testosterone levels in adult mice (*Mus musculus*)

Testosterone is a primary steroid hormone in men derived from cholesterol precursor molecules, produced by the testes. Testosterone stimulates the development of primary sex organs of the embryo and encourages spermatogenesis. Besides, there are three main functions of testosterone, (1) maintaining primary and secondary genitalia, (2) maintaining the process of spermatogenesis to produce spermatozoa, and (3) ensuring the maturation of spermatozoa to be able to carry out fertilization.

The results of this study are supported by previous studies, which show that natural ingredients have an antifertility effect by lowering testosterone levels. One study showed lower testosterone levels in the treatment group of papaya seed methanol extract and papaya seed methanol fraction at a dose of 100 mg/kg body weight[8].The administration of beluntas leaf active compound has the antifertility effect and decreasing testosterone levels[9].

The low level of testosterone in the treatment group is most likely due to low levels of FSH and LH. Ethanol extracts of young papaya seeds inhibit the secretion of FSH and LH simultaneously, which then inhibits the secretion of testosterone by leydig cells or often called interstitial cells.

Apart from inhibiting LH secretion, the ethanol extract of young papaya seeds may have an effect of directly reducing testosterone in leydig cells involving alkaloids. Research has reported that alkaloids and steroids affect the permeability of leydig cell membranes, resulting in food transfer

Table 3. Differences between spermatozoa cell counts in the two groups

Variable	Group	Average ± SD	Median Min-Max	Average difference	CI 95%	p-value
Spermatozoa	Control	75.89±4.71	76.67 67,33±84.00	33.17	30.39-35.94	<0.001*
	Treatment	42.72±3.33	42.50 37.67±49.67			

*p-value<0.05= significant

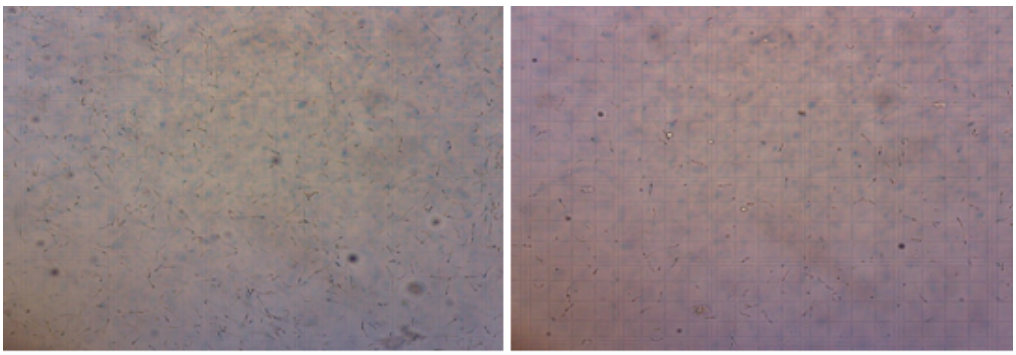


Fig. 1. The number of spermatozoa in the treatment and control group

impairment as an energy source that failed the biosynthesis of testosterone[9].

Alkaloids papaya seed can also inhibit the function and development of leydig cells[10]. Also, alkaloids can inhibit the antioxidant system in leydig cells, thereby disrupting the intracellular redox system and causing disruption of leydig cell physiological functions[11]. When leydig cells are inhibited, the amount of testosterone produced decreases so that the ability of testosterone to stimulate the sertoli cells to carry out the process of spermatogenesis is also reduced.

Ethanol extract of young papaya seeds (*Carica papaya L.*) lower spermatozoa counts in adult mice (*Mus musculus*)

The number of spermatozoa is widely used to test fertility, and also to test the antifertility effect of a particular compound. Previously, papaya seed extract has been extensively studied in terms of its effect as a natural ingredient with antifertility properties.

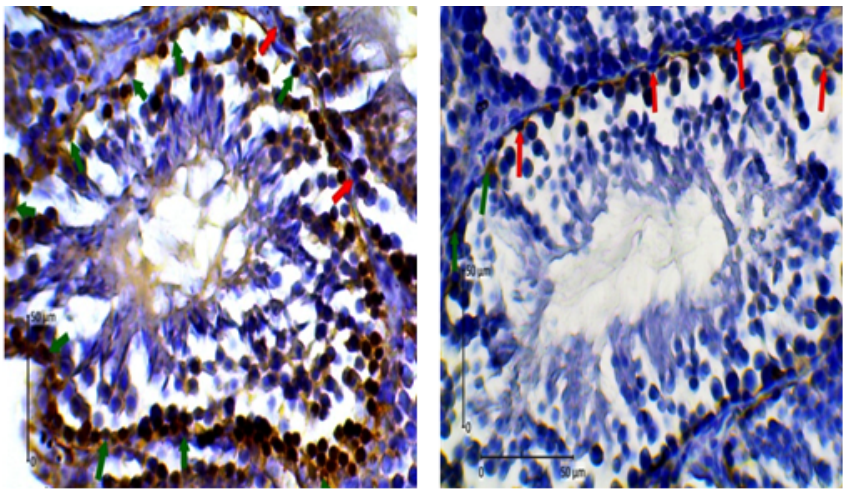
The results of this study explain the antifertility mechanism of young papaya seed ethanol extract comprehensively, ranging from the low levels of FSH and LH, which then leads to low levels of testosterone and low spermatozoa cell counts. Testosterone is needed for the development of germ cells and the release of adult spermatids during stage VIII in mice[12]. FSH, together with testosterone, supports the continuation of meiosis of cells and spermatids through intrinsic and extrinsic pathways[13].

In addition to the barrier effect of spermatogenesis through testosterone, LH, and FSH, the young papaya seed ethanol also contains alkaloids that have a direct cytotoxic effect on spermatozoa. Alkaloids in papaya seeds are known to cause the degeneration of spermatozoa cells and

Table 4. Differences in androgen receptor expression in sertoli cells in both groups

Variables	Group	Average ± SD	Median Min-Max	Average difference	CI 95 %	p-value
Receptor Expression	Control	55.08±2.49	55.60 47.74±59.22	26.97	25.10-28.83	<0.001*
Androgens in Sertoli cells	Treatment	28.11±3.06	27.52 23.81±35.07			

*p-value<0.05= significant



Control Treatment

Fig. 2. Amount of androgen receptor expression in sertoli cells in both groups. Sertoli cell whose cell nucleus has expressed androgens (green arrows); sertoli cell whose cell nucleus has not expressed androgens (red arrows)

reduce the number of spermatozoa cells. It indicates that during the process of spermatogenesis is disordered as a result of the presence of alkaloids in the ethanol extract of young papaya seeds[14].

Ethanol extract of young papaya seeds (*Carica papaya L.*) lower the number of androgen receptor expressions in sertoli cells in adult mice (*Mus musculus*)

The activation of androgen receptor (AR) by testosterone is essential for the maturation and function of leydig cells and the regulation of stereogenic enzymes[15]. Androgen receptor autocrine signaling in leydig cells protects the epithelium of the seminiferous tubules in mice and protects the leydig cell apoptosis in adult mice. The number of receptors in a cell largely determines its reaction to hormones. If enough hormones and receptors are in the cell, the ligand (testosterone) binds and activates the receptor[16].

This study is the first study to examine the effect of young papaya seed ethanol on androgen receptor expression. The reduced expression of androgen receptor to cell types in the testes can be explained as follows: 1) loss of androgen receptor in sertoli cells mainly affects the function of sertoli cells to support germ cells. As a result, spermatogenesis stops in the primary spermatocyte diplotene; 2) due to the reduction of androgen receptor in leydig cells, there is steroidogenesis inhibition so that spermatogenesis stops at the spermatid stage; 3) due to reduced androgen receptor in smooth muscle cells and peritubularmyoid cells there is a decrease in sperm; 4) reduced androgen receptor gene in germ cells does not affect spermatogenesis and fertility[17].

In this study, the expression of androgen receptor was evaluated on sertoli cells; the results imply that young papaya seed ethanol resulted in the cessation of spermatogenesis in diplotene primary spermatocytes[17], resulting in a low number of spermatozoa as previously described. It can be comprehensively asserted that young papaya seed ethanol extract causes infertility (and/or lowers the number of sperm cells) in mice caused by FSH and LH secretion, which leads to lower testosterone and decreased expression of androgen receptor on sertoli cells. Specifically, one mechanism that may be involved in the low expression of androgen receptors in mice given the young papaya seed ethanol is the alkaloids content. Previous studies showed that alkaloids in pepper (*Piper longum*) also decrease the amount of androgen receptors in prostate cancer cell cultures. The study showed that the alkaloids could decrease the expression of full-length androgen receptors or by deletion in the ligand-binding domain (LBD) indicates that domains other than the LBD in androgen receptors that acts as a mediator decreased expression of these androgen receptors. The low number of androgen receptors expression due to alkaloids is probably due to the increased degradation of androgen receptors through the ubiquitin-proteasome pathway[18].

Research Novelty

1. This study is the first to examine the antifertility activity effect of young papaya seed ethanol extract on the level of testosterone and the level of expression of androgen receptors in the sertoli cells of adult mice (*Mus musculus*).
2. This study successfully describes the antifertility mechanism by which young papaya seed ethanol extract complexly mediated through decreased levels of testosterone, which then disrupts cell production of spermatozoa.
3. This study successfully demonstrates that young papaya seed ethanol also induces low levels of androgen receptors on sertoli cells, which also represents another antifertility mechanism of young papaya seed ethanol extract.

CONCLUSION

Young papaya seed ethanol extract can be used as a natural contraceptive in adult mice by decreasing the level of testosterone, spermatozoa count, and the expression of androgen receptors on sertoli cells. It is hoped that human clinical trials will be carried out (in line with applicable rules) to assess if this extract can successfully lower human male spermatozoa counts, and ultimately benefit and support government family planning (KB) programs. Besides, young papaya seed ethanol extract is cost-effective and easy to produce and is likely to be widely accepted within the community.

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None

Conflict of interest

Authors declare there is no conflict of interest

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